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Differentially conserved staphylococcal SH3b_5 cell wall binding domains confer increased staphylolytic and streptolytic activity to a streptococcal prophage endolysin domain

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ABSTRACT

Staphylococcal peptidoglycan hydrolases are a potential new source of antimicrobials. A large subset harbors C-terminal SH3b_5 cell wall binding domains. These C-terminal domains have been shown to be necessary for accurate cell wall recognition and subsequent staphylolytic activity for some endolysins. Over fifty proteins of staphylococcal or phage origin containing SH3b domains were aligned, yielding five highly repetitive groups of proteins. Representative C-termini from these five groups, and six staphylococcal proteins for which no homologues have been identified, were aligned, revealing two distinct SH3b_5 subgroups with overlapping but differentially conserved residues. A premise behind this research is that there may be unique cell wall binding properties conferred by these staphylococcal domains that could be exploited to specifically enhance anti-staphylococcal efficacy in heterologous protein fusion constructs. To identify functional differences between the two subgroups, the native Cpl-7 cell wall binding domains of the streptococcal LambdaSa2 endolysin were replaced by staphylococcal SH3b domains from both subgroups. SH3b domains from either lysostaphin (bacteriocin) or LysK (phage endolysin) resulted in a ~5× increase in staphylolytic activity conferred on the streptococcal endopeptidase domain, and surprisingly these same fusions maintained significant streptolytic activity suggesting that the staphylococcal SH3b domains are not always staphylococcal-specific. A comparison of the differences in lytic activity conferred on the LambdaSa2 endopeptidase domain by either LysK or lysostaphin SH3b domain differed by no more than a factor of two. Through the collection of peptidoglycan hydrolase sequences, three new putative intron-containing phage endolysin genes were identified in public data sets for the phages G1, X2 and 85.

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1. Introduction

SH3 domains (Src homology 3) were first described in eukaryotic proteins that are involved in cell–cell communication and intracellular signaling from the cell surface to the nucleus. These domains favor binding to proline rich sequences (Mayer and Eck, 1995). A comprehensive description of their presence in bacterial proteins was presented (Ponting et al., 1999; Whisstock and Lesk, 1999) and the evolution of protein domain databases has been utilized to identify hundreds of bacterial SH3 (SH3b) domain sequences. Specifically, the Pfam domain database has three groups of SH3b sequences listed in it: SH3_3, SH3_4, and SH3_5 (http://www.sanger.ac.uk/Software/Pfam/) (Finn et al., 2008). Additionally, published SH3b domain sequence alignments reveal that there are conserved residues outside, but in close proximity to, the formal SH3b domains identified (Lu et al., 2006).

The lysostaphin SH3b domain was one of the earliest characterized cell wall binding sequences. A critical 92 amino acid region at the C-terminus of lysostaphin was proved to be essential for staphylococcal cell-type specific binding through fusion of this region to indicator proteins (Baba and Schneewind, 1996). This fusion strategy was also utilized successfully to verify SH3b binding domains for other peptidoglycan hydrolases: LytA (phi11 endolysin) (Baba and Schneewind, 1996), the Ba02 endolysin (Low et al., 2005), Ply500 and Ply118 (Loessner et al., 2002). Recently, Lu et al. purified the *S. capitis* EPK1 lysostaphin homologue, ALE-1, and crystallized its SH3b-containing 92 amino acid C-terminus (Lu et al., 2006).

Abbreviations: ALE-1, *S. capitis* EPK1 lysostaphin homologue; bp, base pair; cDNA, DNA that is complementary to RNA; CHAP, cysteine, histidine dependent amidohydrolases/peptidase; Cpl-7, cell wall binding domain; *lysK*, bacteriophage K endolysin gene; LysK, bacteriophage K endolysin protein; LytA, phage phi11 endolysin; mM, milimolar; N/A, not applicable; ORF, open reading frame; SDS PAGE, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; SH3b, Src homology 3 bacterial cell wall binding domain; λSa2, LambdaSa2 streptococcal bacteriophage; μg, microgram.

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Crystallography proved that this binding domain consists of two molecules, each with eight β-strands arranged in a complex of two anti-parallel multiple-stranded β-sheets (Lu et al., 2006). Naturally occurring serine residue substitutions in the pentaglycine bridge known to confer resistance to both lysostaphin and ALE-1, indicated that the C-terminal ALE-1 92 amino acid region recognizes the peptidoglycan inter-peptide pentaglycine bridge (Lu et al., 2006). This was previously suggested (Baba and Schneewind, 1996) and recently confirmed for lysostaphin via cross-linking studies (Grundling and Schneewind, 2006). These staphylococcal targeting domains do not appear to interact with teichoic acid as do pneumococcal cell wall binding domains (Poxton et al., 1978).

Our study presents the first comprehensive alignment of all SH3b_5 containing staphylococcal sequences that currently reside in public data sets, and collates those proteins that are highly homologous or duplicated into five groups. There are also a handful of 'stand alone' proteins without homologues. When the SH3b domains from the five groups and the stand alone proteins were aligned, they separate out into two distinct subgroups with overlapping but differentially conserved residues. The goal was to identify candidate residues that are highly conserved and thus might be responsible for the near species-specific activity of these putative antimicrobial proteins. In the process of making these sequence comparisons, three new putative intron-containing bacteriophage endolysin genes were identified and their predicted splicing sites described. To test the hypothesis that one or the other subgroup might confer desirable antimicrobial properties to a generic lytic domain, one representative SH3b domain from each of the two subgroups was fused to the recently characterized LambdaSa2 (λSa2) endopeptidase lytic domain (Donovan and Foster-Frey, 2008; Pritchard et al., 2007) and assayed for its contribution to antimicrobial cell lytic properties on both staphylococcal and streptococcal cells.

2. Materials and methods

2.1. Plasmids, constructs and strains

Streptococcal strains include the mastitis isolates *S. agalactiae* and *S. uberis*, gifts from M. Paape, ARS, USDA, Beltsville, MD. Staphylococcal strains included *S. aureus* strain Newbould 305 capsular polysaccharide serotype 5 ATCC 29740), and strain Newman (a gift from Jean Lee, Channing Lab, Brigham Young Womens Hospital).

The streptococcal λSa2 prophage endolysin gene in pET21a (EMD Biosciences, San Diego, CA) was a gift from David Pritchard (Pritchard et al., 2007). C-terminal His-tagged λSa2 deletion constructs in plasmid pET21a were described previously (Donovan and Foster-Frey, 2008). The lysostaphin gene (a gift from David Kerr, Univ. Vermont) and *lysK* gene (Becker et al., 2008) fragments encoding the SH3b domain were amplified with PCR primer pairs: Lyso SalI F (5′-GGA AAA GCA **GTC GAC** ACA GTA ACT CC-3′) and pET21a Sty R (5′-TTA GAG GCC CC-3′) or LysK Xho F (5′-CAAT G**CT CGA G**AG TAC ACC GGC-3′) and pET21a Sty R. PCR amplified fragments were engineered with unique (underlined) N-terminal XhoI or SalI restriction enzyme sites, and unique C-terminal StyI restriction enzyme sites that were designed to allow in-frame gene fusions of these two SH3b domains individually to the C-terminus of the λSa2-E construct in pET21a as described in prior gene fusions (Donovan et al., 2006a; Fig. 4). The λSa2-E construct expresses a 152 amino acid truncation harboring just the λSa2 endopeptidase domain (Donovan and Foster-Frey, 2008). SH3b domain-harboring PCR products were gel purified, digested appropriately with restriction enzymes, purified over a Micro Bio Spin P30 desalting column (BioRAD, Inc.) and introduced into XhoI, StyI digested, dephosphorylated and gel purified λSa2-E vector via conventional means (Sambrook et al., 1989). All constructs are C-terminally modified with eight additional amino acid residues introduced at the C-terminus corresponding to the XhoI site (Leu-Glu) followed by six His residues, and are schematically illustrated in Fig. 4. All subcloning was performed in *E. coli* DH5α (Invitrogen, Carlsbad, CA) for plasmid DNA isolation and sequence verification of all constructs. pET21a constructs were induced in *E. coli* BL21 (DE3) (EMD Biosciences, San Diego, CA).

2.2. Protein purification

Nickel column chromatography purification of His-tagged proteins, truncations, or fusions was per the Qiagen NiNTA protocol (Qiagen, Valencia, CA) as described previously (Donovan and Foster-Frey, 2008).

2.3. SDS PAGE

The purified constructs and Precision Plus protein standards (Bio-Rad) were analyzed with 15% SDS PAGE in Tris-Glycine buffer at 150 V for 1.5 h in Criterion Precast gels (Bio-Rad, Inc., Hercules, CA), according to the manufacturer's instructions.

2.4. Turbidity reduction assays

A standardized turbidity assay modified from (Donovan et al., 2006a) with staphylococcal or streptococcal strains grown to logarithmic phase ($A_{600\ nm}$ = 0.4–0.6) at 37 °C in Brain Heart Infusion broth (DIFCO, Franklin Lakes, NJ) was performed in a 96-well dish and analyzed in a plate reader as described previously (Donovan and Foster-Frey, 2008). As a means to reduce the high variability that often plagues turbidity reduction assay results on live cells, a modified procedure for producing live cell substrate was tested in this work. Eleven liters of log phase cultures of *S. aureus* strain Newman was harvested, pooled, suspended in 110 ml of 10 mM Tris, pH 7.5, 150 mM NaCl, 25% glycerol and frozen at −80 °C in 1.5 ml microfuge tubes, effectively freezing live cells from an identical large-scale culture for use at a later time. As needed for turbidity assays, the frozen cells are thawed, washed 3× via centrifugation with the desired turbidity reduction assay buffer and resuspended in this buffer at the appropriate $OD_{600\ nm}$ ~ 1.0.

2.5. Plate lysis assay

Purified proteins for each construct were sterilized and diluted in either sterile nickel column elution buffer (QIAGEN) or 10 mM Tris, pH 7.5, 150 mM NaCl + 1% glycerol. Ten microliters containing 10, 5 or 2.5 μg was spotted onto a freshly spread lawn of mid-log phase ($OD_{600\ nm}$ ~ 0.4–0.6) of either *S. aureus* or *S. uberis* cells that had air-dried for 10 min on tryptic soy agar plates. The spotted plates were air-dried for ~ 10 min in a laminar flow hood, and incubated overnight in a 37 °C environment. Scoring of the cleared spots occurred within 20 h of plating the cells.

2.6. Bioinformatics: defining groups of homologous proteins

To align and identify homologues among the staphylococcal proteins containing SH3b_5 domains:: PFAM: http://www.sanger.ac.uk/Software/Pfam/ (Finn et al., 2008); NCBI: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search and DB=Nucleotide; SMART: http://smart.embl-heidelberg.de/ (Schultz et al., 2000); BLAST: http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al., 1990); CLUSTAL: http://www.ebi.ac.uk/clustalw/index.html# (Larkin et al., 2007); GCG Wisconsin DNA sequence analysis Seqweb Version 3.1: seqweb.ncifcrf.gov (Accelrys). In order to allow this summary of protein sequences to serve as a resource to identify duplications of the staphylococcal SH3b_5 peptidoglycan hydrolase proteins in Genbank, no effort was made to remove duplicate entries from the initial

Table 1
Groups of staphylococcal C-terminal SH3b domain containing proteins.

		Length aa	Identity within group	Accession #
Group I				
1	phiNM1	484	>90%	ABF73094.1
2	phiNM4	484	>90%	ABF73293.1
3	ROSA	484	>90%	AAX91502.1
4	phage53	484	>90%	AAX90838.1
5	phage77	484	>90%	NP_958622.1
6	prophageL54a	484	>90%	AAW38858.1
7	amidaseMRSA252	484	>90%	CAG40495.1
8	phiSLTORF484-like	484	>90%	ABD20547.1
9	phiSLT	484	>90%	NP_075522.1
10	phi12	484	>90%	NP_803355.1
11	phage47	484	>90%	AAX91198.1
12	hydrolaseaureusNCTC8325	484	>90%	ABD30597.1
13	phiPVL_108	484	>90%	YP_918945.1
14	truncamidaseaureusMW2	484	>90%	NP_646197.1
15	phage3A	484	>90%	YP_239959.1
16	phagePVL	484	>90%	NP_058463.1
17	phage96	484	>90%	YP_240259.1
18	amidaseMSSA476	484	>90%	YP_043081.1
19	phage80alpha_new[a]	484	>90%	ABF71642.1
20	phagetp310-2	484	>90%	YP_001429961
21	amidase USA300_TCH1516	484	>90%	ABX29441
22	phagetp310-1	484	>90%	YP_001429893
Group II				
1	phage80alpha[a]	481	>90%	AAB39699.1
2	phi11orf53[b]	481	>90%	NP_803306.1
3	autolysinaureusNCTC8325[b]	481	>90%	YP_500516.1
4	phi11LytA_Autolysin[b]	481	>90%	P24556
5	phage29	481	>90%	YP_240560.1
6	phage52A	481	>90%	YP_240634.1
7	phage55	481	>90%	YP_240484.1
8	phage69	481	>90%	YP_239596
9	amidaseJH1	481	>90%	YP_001316244
10	amidaseJH9	481	>90%	YP_001246457
11	phage92	481	>90%	YP_240773.1
12	phiNM2	481	>90%	ABF73160.1
13	amidaseaureusMu50	481	>90%	NP_371437.1
14	phage88	481	>90%	YP_240699.1
15	phagephiMR11	481	>90%	YP_001604156
16	phage85orf21/18[c]	481	>90%	AY954953
17	phage-relatedamidaseRF122	481	>90%	YP_417165.1
18	phageX2orf18/19[c]	481	>90%	NC_007065
19	amidase|str.Newman	481	>90%	YP_001332073
20	phiMR25	481	>90%	YP_001949866
21	amidase2JH1	481	>90%	YP_001315525
22	amidase2JH9	481	>90%	YP_001245749
23	phage80	481	>90%	ABJ88906
24	amidaseMu3	481	>90%	YP_001441498
25	phageEW[d]	481	76%	YP_240182
26	phage37[d]	481	83%	YP_240103
Group III				
1	phageK	495	>99%	YP_024461
2	phage812[c]	494	>99%	ABL87139
3	phageG1orf42/60	495	>99%	AY954969
Group IV				
1	phage66	250	>90%	YP_239469.1
2	phage44AHJD	250	>90%	NP_817310.1
3	phagephiP68	250	>90%	NP_817332.1
4	phageSAP-2	250	>87%	YP_001491539
Group V				
1	matureLysostaphin	246	>80%	P10547
2	matureALE-1[e]	244	>80%	BAA13069.1
Stand alone proteins				
1	phage37[d]	481	N/A	YP_240103
2	phageEW[d]	481	N/A	YP_240182
3	phage 2638A	486	N/A	AAX90995
4	phage twort	467	N/A	AAX92311
5	haemolyticus JCSC1435	494	N/A	BAE05642.1
6	phiWMY	477	N/A	BAD83402.
Finalist list				
1	phage55	481	N/A	YP_240484.1
2	phage37	481	N/A	AAX91269
3	phageEW	481	N/A	AAX91347
4	phagephiP68	250	N/A	NP_817332.1
5	phage twort	467	N/A	AAX92311
6	phiWMY	477	N/A	BAD83402.
7	haemolyticus JCSC1435	494	N/A	BAE05642.1
8	phageK	495	N/A	YP_024461
9	phage 2638A	486	N/A	AAX90995
10	matureLysostaphin	246	N/A	P10547
11	prophageL54a	484	N/A	AAW38858.1

N/A — not applicable.

[a] In Group I, the phage 80α (new) (ABF71642.1) protein sequence was derived from phage genomic DNA (Genbank, direct submission; DQ517338) and likely describes the correct phage 80α endolysin sequence. In Group II, the sequence phage 80α protein (AAB39699.1) is nearly identical (one amino acid difference) to the phi11LytA_Autolysin (P24556) sequence that was recently shown with MALDI-TOF to be erroneous (Donovan et al., 2008). The genomic DNA sequence from which the phage 80α protein (AAB39699.1) sequence was generated, has the identical pair of sequencing errors that caused a frame shift in the predicted open reading frame of phi11LytA_Autolysin (P24556) and was also generated from bacterial genomic sequences, from *S. aureus* NCTC 8325 (direct submission; Bon, J.B., Mani, N. and Jayaswal, R.K.).

[b] In Group II, the proteins phi11ORF53 (NP_803306.1), phi11LytA _Autolysin (P24556) and autolysin *aureus* NCTC 8325 (YP_500516.1) are most likely derived from the same genes. They share the same flanking genomic sequences, and aside from translational start site assignment errors and sequencing errors (including two that cause frame shifts in the protein coding sequences), are virtually identical. The sequence and ORF assignment discrepancies have been clarified previously (Donovan et al., 2008). The correct sequence is autolysin *aureus* NCTC 8325 (YP_500516.1).

[c] Newly identified putative intron-containing coding regions.

[d] Phage 37 and phage EW endolysins are from Group II, but were placed additionally in the stand alone category due to the overwhelmingly higher level of conservation between the other members of Group II.

[e] Mature ALE-1: the Pre-pro sequence of lysostaphin is removed by *S. simulans* during maturation of the protein (Recsei et al., 1987). The homologous sequences are not all removed from ALE-1 by *S. capitis* (Sugai et al., 1997). To highlight the homology between the family members, a hypothetical "mature ALE-1" is presented in Supplemental data, which compares a truncated ALE-1 (N-terminal sequences artificially removed) to highlight the similarity with the lytic and SH3b domains of lysostaphin.

alignments in the Supplemental data, and Table 1, unless specifically described in the text.

3. Results

3.1. Sequence comparison of staphylococcal SH3b_5 domain-harboring proteins

BLAST analysis using known staphylococcal phage endolysin protein sequences were utilized to identify five groups of homologous proteins with >90% within group identity listed in Table 1 (group alignments are in Supplemental data). Group I, II, and III proteins were organized with N-terminal cysteine, histidine dependent amidohydrolases/peptidase (CHAP) domain (Bateman and Rawlings, 2003) followed by an amidase domain (Rigden et al., 2003) and a C-terminal SH3b domain (Fig. 1).

Group IV and V proteins have only one lytic domain (CHAP endopeptidase or glycyl–glycine endopeptidase domain, respectively) and a C-terminal cell wall binding domain (Fig. 1). CHAP domains are on average 24% conserved within the PFAM data base. Within group, CHAP domains are highly conserved (>90% identity, excluding stand alone group) but show less conservation between groups. The highest within group identity exists between Group I and Group II, less than 52% identity. All other groups show less than 50% identity between CHAP domains. The Amidase domains are less similar across groups. The central domains in proteins from Group I are identified as Amidase_3 domains and do not share significant sequence identity with the Amidase_2 domains identified in Groups II and III, which share ~53% identity. Exact assignment of CHAP, amidase and SH3b

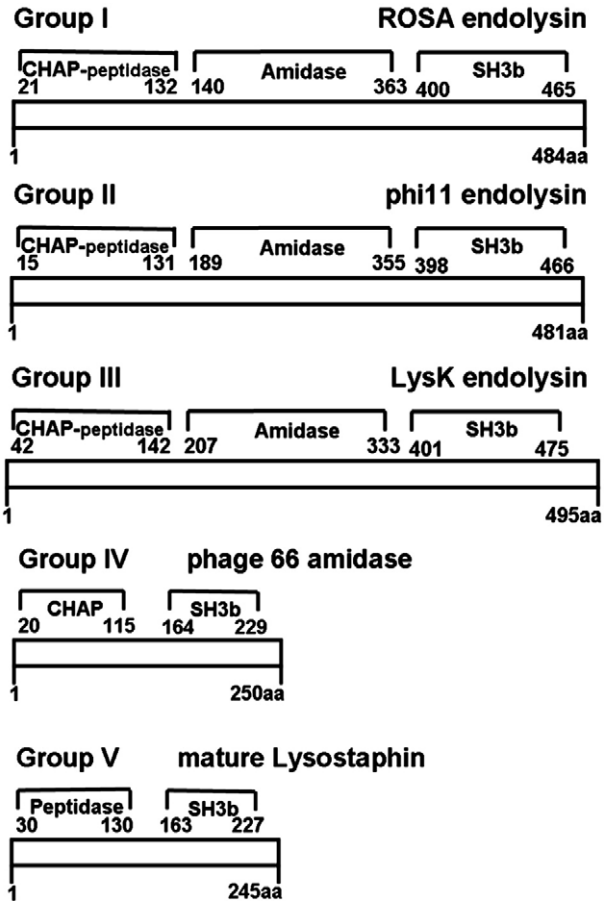

Fig. 1. Schematic description of representative proteins from each of the five groups of SH3b_5 containing staphylococcal proteins. Nomenclature and accession number of each protein is from Table 1.

sequences is available through Genbank via the accession number (Table 1). There are also six proteins in a "Stand Alone" category (Group VI) that are less than 50% identical to other groups. Inter-group comparisons reveal <50% between-group identity. Phage 37 and phage EW endolysins are from Group II, but were placed in the stand alone category due to the overwhelmingly higher level of conservation between the other members of Group II.

3.2. Clarifying database sequence discrepancies

Sequence discrepancies were identified in open reading frame (ORF) assignments for three Group II entries: phi11ORF53 (NP_803306.1), phi11LytA (P24556), and the *S. aureus* 8325 autolysin (YP_500516) that are discussed in the legend of Table 1, and were recently resolved (Donovan et al., 2008). Only the correct version, *S. aureus* 8325 autolysin (YP_500516), was included in the sequence alignment (Supplemental data). Other discrepancies exist in public data sets for the phage 80 alpha endolysin (described in the legend of Table 1). The original phage 80 alpha amidase sequence (phage 80 alpha; AAB39699.1) with 481 amino acids placed the protein in Group II. A more recent Genbank entry with 484 amino acids [phage 80 alpha (new); ABF71642.1] places the protein in Group I. It is likely that the phage 80 alpha (new) (ABF71642.1) is the correct endolysin sequence, and was the only phage 80 alpha sequence included in the alignments (Supplemental data). DNA sequence errors are not uncommon in public databases, and in fact are estimated at 0.01% (Wesche et al., 2004). All public database entries are maintained in Table 1, to alert the reader, but only the correct sequences were utilized in the Supplemental data alignments.

3.3. Putative intron-containing genes

BLAST alignments indicated that several of the endolysin genes in this study likely contain previously unreported introns. Both Group II and Group III BLAST analyses yielded open reading frames (ORFs) in phage 85, X2 and G1 of partial endolysin sequences with nearly 100% identity to respective group members. Group II homologies were phage 85 ORFs 21 and 18 and phage X2 in ORFs 18 and 19. Group III homologies were phage G1 ORFs 42 and 60. Further analysis (Supplemental data) led to the predicted sequences utilized for alignments in Table 1 and in the Supplemental data. The predicted protein sequence has not been verified via cDNA sequence analysis.

Groups III and IV (Table 1) have high 'within group' conservation, and originate from nearly identical phages (Eyer et al., 2007; Vybiral et al., 2003). The *lysK* cDNA sequence was obtained by reverse transcription of *lysK* mRNA (O'Flaherty et al., 2005); as such, the LysK protein sequence was used to predict the G1 endolysin protein sequence (Supplemental data). The predicted phage 812 endolysin sequence has a single amino acid deletion that corresponds to the intron splice site at codon 66 despite 100% identity among the Group III endolysins at every other residue. There is no evidence that the phage 812 endolysin splice site was determined via cDNA sequence analysis. Thus, it is possible that this single amino acid omission is due to alternative RNA splice sites, but it is most likely due to incorrect assignment of the phage 812 endolysin mRNA splice sites.

3.4. Aligning C-terminal SH3b containing sequences

A representative member of each of the five groups and each of the six stand alone proteins (finalist list, Table 1) were compared in CLUSTAL (default settings; Supplemental data). The C-terminal region that extends ~9 residues N-terminal and ~22 residues C-terminal to the formal 63 amino acid SH3b domain (as defined by the PFAM conserved domain database; over lined in Fig. 2) had the greatest homology in this comparison. This region corresponds nearly exactly to the 92 amino acid C-terminal cell wall binding region described previously for both lysostaphin (Baba and Schneewind, 1996) and ALE-1 (Lu et al., 2006). The finalist C-terminal sequences were next aligned separately (Fig. 2) to remove any alignment bias created by the full protein sequences. The alignment of the eleven finalist C-terminal regions identified seven perfectly conserved amino acid positions, 12 highly conserved and eight weakly conserved residues. Most of these conserved residues are within the formal SH3b domain, but one perfectly conserved Gly residue and five other highly conserved residues are outside of the formal SH3b region. Previous site-directed mutagenesis studies demonstrated that amino acid residues outside of the SH3b domain for both ALE-1 (Lu et al., 2006) and lysostaphin (Donovan: unpublished data) are essential for full binding and full staphylolytic activity, respectively.

CLUSTAL alignment program also depicts inferred ancestral relationships between the sequences through cladograms. In Fig. 2B, the 11 unique C-terminal domains show a greater relatedness among six of the seven proteins (top half of cladogram: phage 55, phage 37, phage EW, phage phi P68, phage K and prophage L54a) than the remainder of the sequences. The top half of the cladogram represents Groups I–IV (including the low identity Group II members, phage EW and phage 37) where as the lower half of the cladogram is composed of the stand alone members from Group V. To further examine these relationships, CLUSTAL alignments were performed separately on the top and bottom half of the cladogram. Top half alignment indicated 10 perfectly conserved amino acid positions (Fig. 3A), while bottom half alignment indicated 32 perfectly conserved residues (Fig. 3B). To test whether or not the higher conservation in the bottom half of the cladogram was due to the reduced stringency of aligning fewer sequences, the alignment for the top half was repeated with only four sequences. Phage 37 and phage EW protein sequences were removed

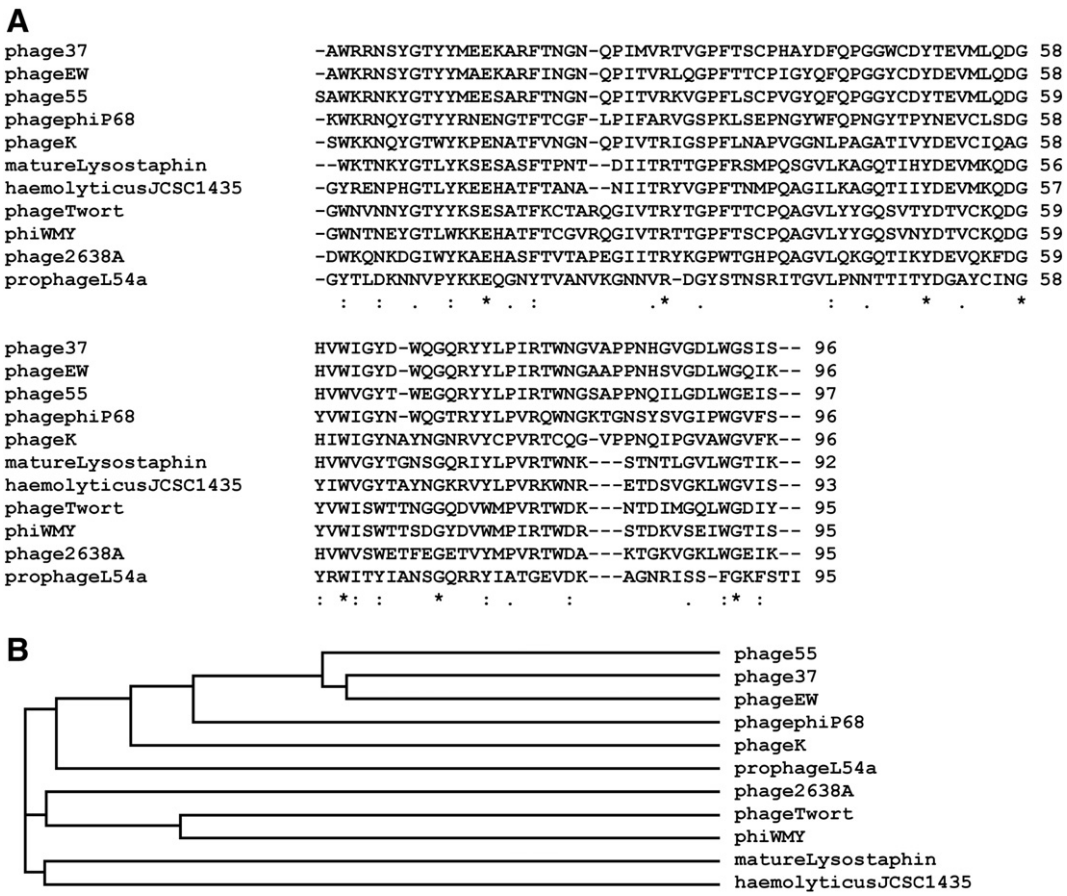

Fig. 2. Conservation of amino acid residues in the cell wall binding domains of the eleven unique finalist proteins. (A) Clustal alignment of representative C-terminal sequences from Groups I–V and the stand alone proteins. Overlined residues constitute the formal SH3b_5 domain (Pfam database). Stars = identity; colon = highly conserved amino acid side chain; single dot = weakly conserved amino acid side chain. (B) Cladogram analysis of the C-terminal regions of the eleven finalist proteins depicted in panel A.

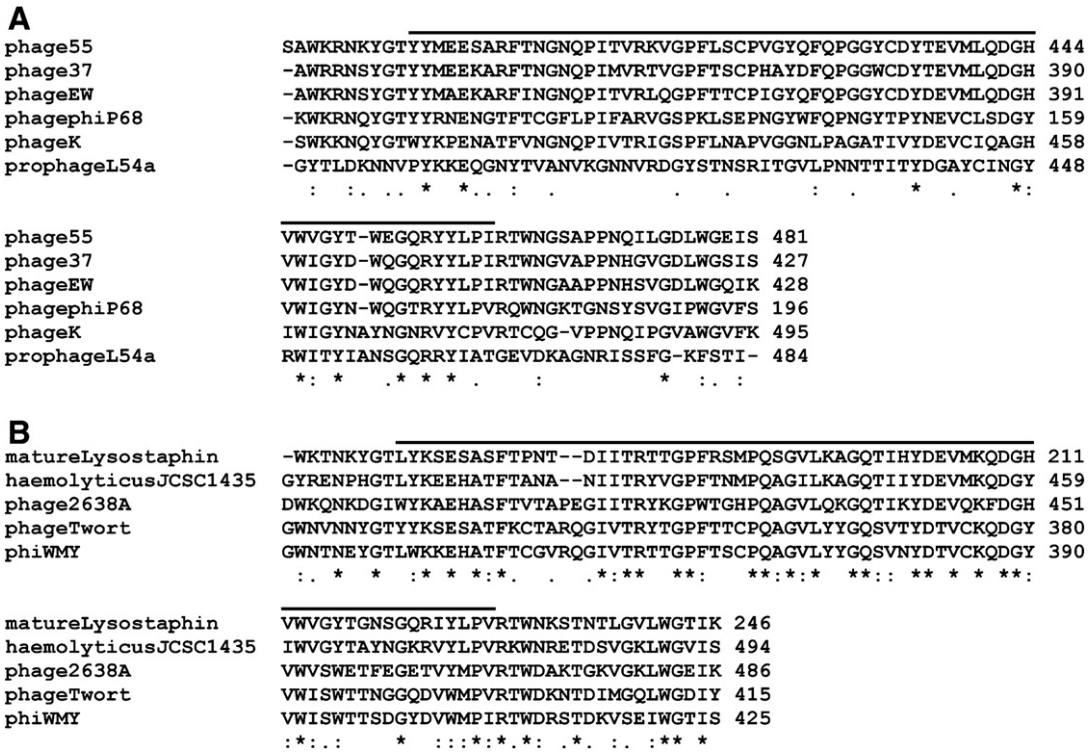

Fig. 3. Subgroup conservation of amino acid residues in the C-terminal sequences of the eleven finalist proteins. Overlined residues constitute the formal SH3b_5 domain (Pfam database). (A) Weakly conserved subgroup. (B) Highly conserved subgroup.

from this test alignment due to their similarity to the phage 55 protein. The level of conservation from the alignment with all six sequences (Fig. 3A) was identical to the alignment with just four sequences (data not shown).

3.5. Testing staphylococcal SH3b domains for their contribution to antimicrobial efficacy

The streptococcal λSa2 prophage endolysin has strong lytic activity for multiple streptococcal pathogens and weak lytic activity for some staphylococcal pathogens (Donovan and Foster-Frey, 2008). This is likely explained by the site of λSa2 endopeptidase cleavage (between L-Lys and D-Ala; Pritchard et al., 2007) being conserved in the stem peptide of peptidoglycan from both genera (Schleifer and Kandler, 1972). A schematic of the λSa2 parental protein and pertinent deletion/fusion constructs are illustrated in Fig. 4A. SDS PAGE analysis of the resulting nickel chromatography purified proteins from each construct indicates >90% purity of each (Fig. 4B). Prior work demonstrated that the endopeptidase domain lytic activity was reduced ~10× in the absence of the Cpl-7 cell wall binding domains

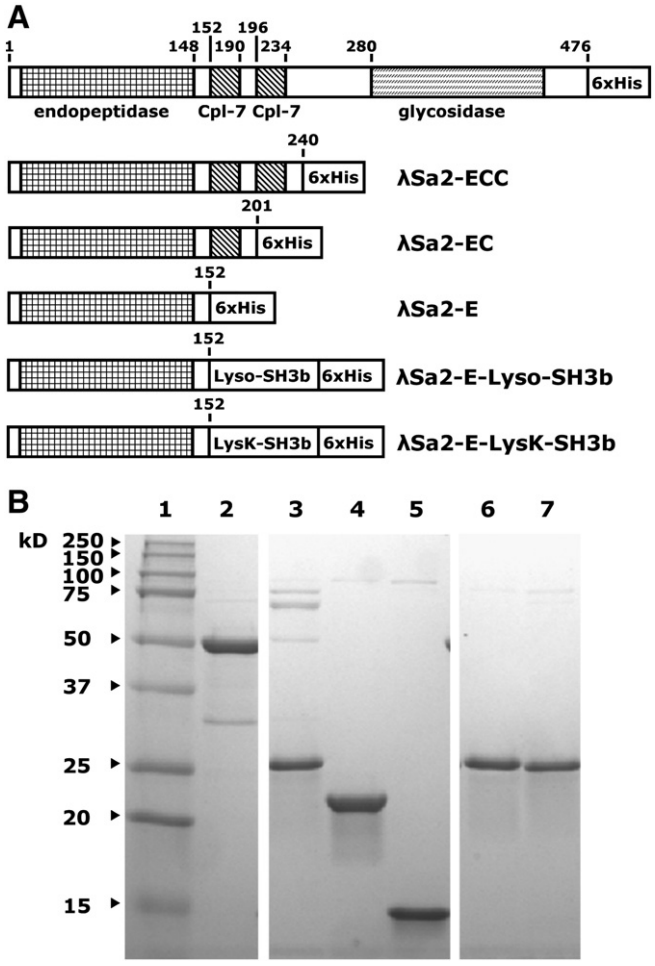

Fig. 4. Schematic representation and SDS PAGE analysis of the His-tagged full length, truncations and fusion constructs of the λSa2 endolysin. (A) Full-length λSa2 endolysin, λSa2-ECC, λSa2-EC and λSa2-E have been described previously (Donovan and Foster-Frey, 2008). The fusion junction points of the λSa2 endopeptidase domain to both of the staphylococcal SH3b sequences occurs at amino acid 152 of the λSa2 protein, just prior to the Cpl-7 sequences. Each SH3b domain-harboring fragment includes the entire C-terminus of the parental protein (lysostaphin or LysK) starting 10 amino acids prior to the 63 amino acid SH3b domain (per Pfam database). (B) SDS PAGE analysis of 5 μg nickel column purified proteins. Lane 1, markers; lane 2, λSa2 endolysin; lane 3, λSa2-ECC; lane 4, λSa2-EC; lane 5, λSa2-E; lane 6, λSa2-E-Lyso-SH3b; lane 7, λSa2-E-LysK-SH3b. Endolysin sequences and SH3b sequences are drawn nearly to scale. His tags are not drawn to scale.

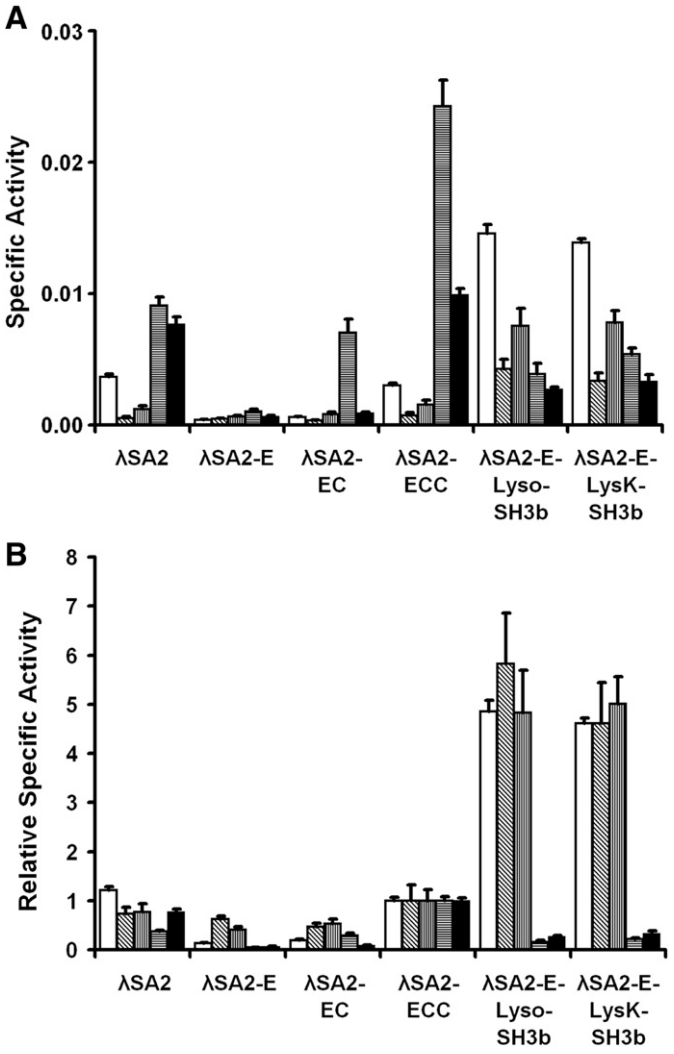

Fig. 5. Turbidity reduction analysis with λSa2 constructs on streptococcal and staphylococcal cells. (A) Specific activities ($\Delta OD_{600\ nm}$/min/μg) for the λSa2 deletion and fusion constructs with three staphylococcal cell preparations and two streptococcal cell preparations. Fig. 4 construct names are utilized. (B) Normalized Specific Activities for Panel A data. The specific activities for each construct were normalized to the λSa2-ECC construct in order to better visualize the effect resulting from the SH3b fusion to the λSa2-E domain. Open Bars, frozen *S. aureus* strain Newman; diagonal Stripes, fresh cultured *S. aureus* strain Newman; vertical stripes, fresh cultured *S. aureus* strain 305; horizontal stripes, *S. agalactiae*; black bars, *S. uberis*. Each data point (+/−SEM) represents triplicate samples at 100 μg/ml from at least two unique protein preparations with at least two experiments performed on each ($n \geq 12$).

(λSa2-E, Fig. 4.) When the endopeptidase construct contained just the two native Cpl-7 cell wall binding domains (λSa2-ECC, Fig. 4), its lytic activity increased on both streptococcal and staphylococcal species to a level greater than the full-length λSa2 protein (Donovan and Foster-Frey, 2008). It was hypothesized that by replacing the native Cpl-7 domains with staphylococcal SH3b domains (one from each subgroup identified in Fig. 3; e.g. LysK vs. lysostaphin), measurements of lytic activity from these heterologous fusions could be exploited to compare the two subgroups of staphylococcal SH3b domains for their relative ability to enhance staphylolytic activity (Fig. 5). The LysK and lysostaphin SH3b domains were chosen for fusion to the streptococcal endopeptidase domain for several reasons: 1. They are from opposing subgroups of SH3b domains (highly conserved vs. weakly conserved groups, as depicted in Fig. 3); 2. They represent two types of proteins—a bacteriocin (lysostaphin) vs. a phage endolysin (LysK); and 3. They share less than 50% identity at the amino acid sequence level.

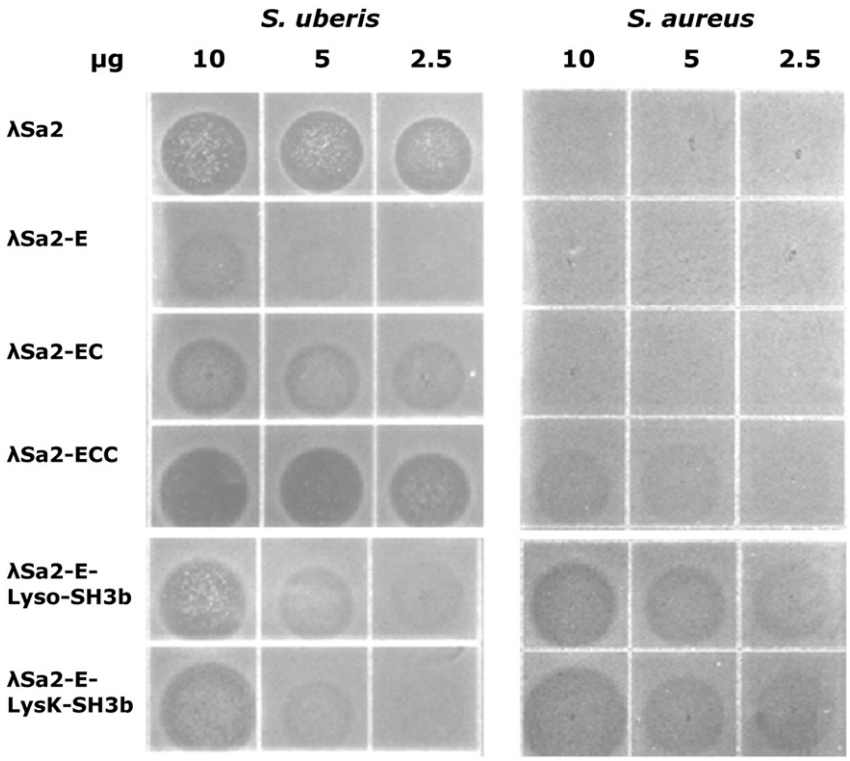

Fig. 6. Plate lysis analysis of λSa2 derived proteins with *S. uberis* and *S. aureus*. Either 10, 5 or 2.5 μg of sterile pure protein in a10 μl volume of buffer was spotted onto a freshly plated (TSA plates), air-dried, lawn of mid-log phase bacteria cultured in TSB. Plates are read after overnight incubation at 37 °C. Cleared spots represent lawn inhibition due to cell lysis. Buffer alone controls (not pictured) do not alter lawn growth.

Multiple strains of both *S. aureus* (strain Newman and Newbolt 305) and streptococcal species (*S. agalactiae*, *S. uberis*) were tested in the turbidity reduction assay by the heterologous fusions to avoid bias due to strain-specific responses that are often associated with peptidoglycan hydrolase activity measurements (Kusuma and Kokai-Kun, 2005). Turbidity reduction assay results with 100 μg/ml of each λSa2-derived constructs are presented in Fig. 5A. However, to more clearly illustrate the ~5× increased staphylolytic effect of the SH3b domain fusions, the specific activity of each construct was normalized to the specific activity of λSa2-ECC and presented in Fig. 5B. Results of these turbidity reduction assays with λSa2, λSa2-E, -EC, and -ECC constructs agree with previous turbidity reduction assay results (Donovan and Foster-Frey, 2008).

In order to verify these findings in a second type of assay, plate lysis assays (Fig. 6) were performed with *S. uberis* and *S. aureus* identical protein preparations used in the turbidity reduction assays in Fig. 5. The patterns of plate lysis mimic the turbidity reduction assay results as well as previous reports (Donovan and Foster-Frey, 2008). The λSa2-ECC construct demonstrates robust lysis for streptococci in the plate lysis assay. Both the lysostaphin- and LysK-SH3b fusion constructs demonstrate a nearly identical pattern of increased staphylolytic activity compared to the λSa2-ECC construct in the plate lysis assay, but also indicate a level of clearing against streptococcal cells comparable to that of the λSa2-EC construct.

In the plate lysis analysis (Fig. 6), the λSa2-E-Lyso-SH3b shows clearing of *S. uberis* in the 2.5 μg spot while λSa2-E-LysK-SH3b does not. In contrast, there was not a similar end point of lytic activity identified with plate lysis for *S. aureus*. Both fusion constructs show full lysis of *S. aureus* at the lowest concentration tested (2.5 μg/10 μl). Thus, to examine the relative activity of these two constructs on *S. aureus* at limiting concentrations, a turbidity reduction assay was performed (Fig. 7). The λSa2-E-Lyso-SH3b construct is consistently more active than the λSa2-E-LysK-SH3b construct at all points along the plot of activity vs. increasing concentrations. The effect is not more than 2 fold, but the difference is statistically significant when the rates of turbidity reduction are compared at paired protein concentrations. (Fig. 7A) The difference is also apparent when rates are compared on a molar basis (Fig. 7B). In contrast, the wild type LysK and lysostaphin samples in these same turbidity reduction assays show the inverse in relative activity with Lysostaphin consistently showing less activity than LysK, with not more than 2 fold differences noted. It should also be noted that the relative activity of LysK vs. Lysostaphin is highly dependent on salt concentration as described previously by Becker et al. (2008), although at this 150 mM NaCl concentration, their specific activities are not statistically different at higher protein concentrations (20 and 40 μg/ml).

4. Discussion

There is conflicting evidence in the literature defining the role of the SH3b domain in peptidoglycan hydrolase proteins. The evolutionary conservation of these domains indicates an added value for their maintenance, but experimental data does not always fully support that inferred role. The SH3b domain for some proteins is essential for correct species-specific targeting of the endopeptidase domain to *S. aureus* peptidoglycan, e.g. lysostaphin (Baba and Schneewind, 1996) and ALE-1 (Lu et al., 2006). This is also true for the cell wall binding domains of some phage lytic enzymes from *Listeria monocytogenes* (Loessner et al., 2002). However, there are numerous reports of C-terminally deleted lysins where the N-terminal lytic domain maintains staphylococcal- (Donovan et al., 2006c) or streptococcal-specificity (Donovan et al., 2006a; Donovan et al., 2006b) in the absence of its SH3b domain. Also, at high enough concentrations, even lysostaphin will digest the peptidoglycan of staphylococcal species other than *S. aureus* (Cisani et al., 1982).

A sequence comparison of the known staphylococcal SH3b_5 harboring proteins identified five highly conserved groups of peptidoglycan hydrolase proteins and a group of six ‘stand alone’

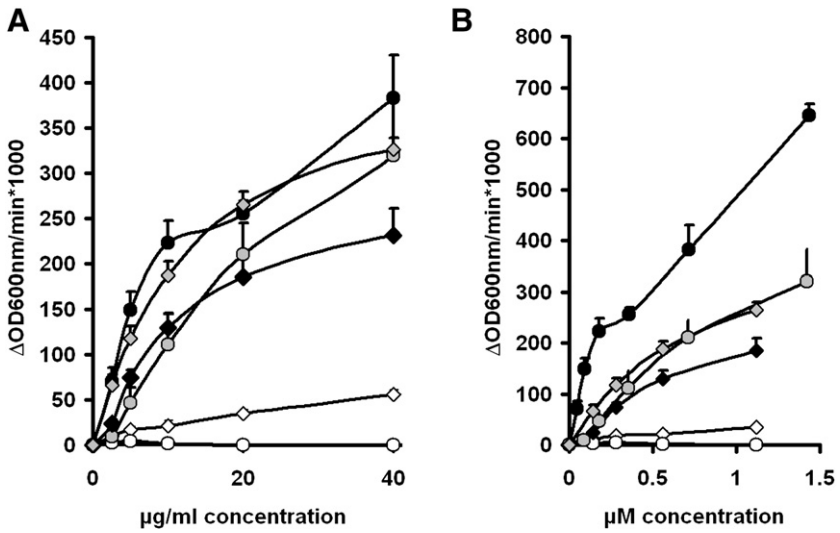

Fig. 7. Linear range determination for turbidity reduction lytic activity of λSa2 derived proteins on *S. aureus*. Turbidity reduction assays were performed with LysK (black circles), Lysostaphin (grey circles), λSa2-E (open circles), λSa2-ECC (open diamonds), λSa2-E-LysK-SH3b (black diamonds), and λSa2-E-Lyso-SH3b (grey diamonds). The same data is calculated either as μg/ml of enzyme (A) or μmol of enzyme (B). Serial 2 fold dilutions of each enzyme were tested for activity against *S. aureus* strain Newman cells. Assays were performed in 150 mM NaCl, 10 mM tris, 0.1% BSA, pH 7.5. Each value represents the highest change in activity observed over a 40 second interval during the 5 minute plate reader assay. Results from the serial dilution analysis were subjected to two way anova, comparing both the enzymes and differing enzyme concentration. All effects were found to be statically significant at the alpha 0.05 level. Preplanned comparisons between the truncation protein λSa2-ECC and the fusion proteins λSa2-E-LysK-SH3b and λSa2E-Lyso-SH3b were performed using the Student's *t*-test (alpha 0.05). This analysis found the truncation protein λSa2-ECC is significantly different than either fusion at all paired protein concentrations, except λSa2-ECC vs λSa2-E-Lyso-SH3b at the lowest concentration tested 2.5 μg/ml. Additionally, the fusions with LysK or lysostaphin SH3b domains have significantly different rates at the highest three concentrations tested, 40, 20 and 10 μg/ml. Error bars represent the standard error of the mean of a minimum of 3 trials, each composed of three replicates.

proteins, for which no highly conserved homologues have yet been reported. All of the SH3b_5 containing proteins contain an endopeptidase domain, with the CHAP endopeptidase being the most common. The dual lytic domain proteins all have an N-terminal CHAP endopeptidase, mid-protein amidase domain and C-terminal SH3b_5 domain. Alignment of representative C-terminal sequences (one from each group and the six 'stand alone' proteins), identified seven perfectly conserved amino acid residues and numerous highly conserved residues. The conservation is limited to the C-terminal 92 amino acids, with conserved residues located both within and outside of the formal 63 bp SH3b_5 domain. Some of these domains have been shown with functional assays to be important for binding (Lu et al., 2006) or full lytic activity (Donovan: unpublished data). The conserved residues constitute a consensus sequence for staphylococcal cell wall binding domains that differ from prior reports of multi-species SH3b domain alignments (Lu et al., 2006; Ponting et al., 1999). Cladogram analysis was used to identify two major subgroups within the staphylococcal SH3b_5 domains, each with a different level of conservation.

It is noteworthy that there were no lysozyme-like glycosidases discovered in this comparison of staphylococcal peptidoglycan hydrolases. Lysozyme-like glycosidase domains are known to exist in SH3b-containing bacteriophage endolysins from Gram positive species (Baker et al., 2006; Porter et al., 2007), and are also believed to exist in the staphylococcal genome, presumably to play a role in the cell wall synthesis and degradation necessary for staphylococcal cell growth and division (Boneca et al., 2000; Komatsuzawa et al., 1997). A variety of lysozymes of bacterial origin are also known to exist (Nakimbugwe et al., 2006). Although most are not able to digest staphylococcal peptidoglycan, due to the ability of some staphylococci to O-acetylate their peptidoglycan (Bera et al., 2006), there are other lysozymes that can (Brau et al., 1991). In light of the fact that phage genomes are notoriously recombinogenic, it is difficult to reconcile why there is not a single glycosidase domain among the SH3b containing phage or staphylococcal peptidoglycan hydrolases.

The evolution of some polypeptide domains has been postulated to involve introns (Schmidt and Davies, 2007). The high degree of homology in phage G1, 812 and K genomes, and the presence of introns in both phage K and 812 endolysin genes, suggest that the G1 endolysin gene might also contain an intron. Although there is no report that these (putative) intronic ORFs actually encode functional proteins, the known and putative introns of the genes encoding Group III proteins and Group II (Table 1) phage 85 and X2 endolysin genes contain an ORF [phage G1, #AAO47478.1; phage 812, #ABL87140; phage K, # YP_241095.1); phage X2; #YP_240848; phage Y2, #AAX90936.1)] that shares homology with the HNH homing endonuclease family of proteins, as would be predicted for Group I introns (Haugen et al., 2005). With none of the other Group II protein genes containing introns, it was unpredicted that the highly homologous phage 85 and X2 endolysin genes would each contain putative introns.

A recent depiction of the staphylococcal phage proteomic tree (Daniel et al., 2007), places the phages X2 and 85 within a branch of the *Siphoviridae* that is shared with other phage which do not have intron-containing endolysin genes (phages 55, 29, 52A, 92, 88, EW, 37). The presence of putative introns in just two of the Group II proteins (Table 1), if verified, would further raise questions about the role that these putative genetic elements might play in the evolution of these genomes. Introns have been proposed to participate in the creation of novel polypeptide domains through the process of exonization of intronic sequences or exon shuffling (Schmidt and Davies, 2007). The location of the putative introns in the Group II endolysin genes position long stretches of putative intronic sequences (~930 nt.) between two distinct lytic domains, potentially increasing the possibility of recombination in this genomic region, increasing the chance of exon shuffling.

The predicted endolysin gene introns of phage X2 and 85 interrupt the mRNA either at codon 203 or 204. This is just a few codons away from the start of the amidase domain, which extends from amino acid 200 to 320 (Fig. 2). This putative gene structure effectively isolates the CHAP endopeptidase and the amidase domains on separate exons. Each phi11 endolysin lytic domain is functional when isolated (Donovan et al., 2006c; Sass and Bierbaum, 2007). If this data can be extrapolated to the >90% identical phage 85 and X2 endolysins from this same group, then the putative introns are effectively isolating the coding sequences for two functional lytic cassettes

(CHAP vs. amidase-SH3b) on separate exons. It is interesting to speculate that an intron-assisted recombination event brought these lytic domains to their current positions in their respective phage genomes. This pattern is consistent with the (putative) exon 1 (encoding a CHAP domain) of the Group III protein genes (Table 1 and Supplemental data) but does not extrapolate to exon 2 of the Group III genes where the (putative) intron interrupts the sequences encoding the amidase domain at codon 266, near the center of this second lytic domain. It is interesting that no (putative) introns are predicted at a position that would separate the sequences encoding a lytic domain from its SH3b domain.

Having identified two subgroups among the staphylococcal SH3b domains, we hypothesized that a bacteriocin (lysostaphin) might harbor an SH3b domain that confers better antimicrobial properties than a phage endolysin (LysK). For example, a cell wall binding domain with a weaker binding constant might allow a lysin to move rapidly between cells. This could make a more effective antimicrobial by allowing the protein to lyse more cells in a shorter period of time. A strong binding domain with a reduced off-rate might, in the extreme case, bind to just one cell and not be released. Such a binding domain might limit the lysin (phage K) to killing just one cell, in order to allow the phage to escape, but not lyse potential host cells in the vicinity, thus reducing its overall antimicrobial impact on an active infection (Martin Loessner, personal communication). To compare the effect of these two domains, the fusion constructs were tested both in turbidity reduction assays and plate lysis assays. Although it was our goal to identify a cell wall binding domain that might confer a much higher staphylolytic activity, in this heterologous fusion analysis we were only able to show that the lysostaphin SH3b domain could confer a ~2× increase in lytic activity over the LysK SH3b domain.

Heterologous fusions have been used previously to analyze the contribution of cell wall binding domains, most notably between clostridial and pneumococcal peptidoglycan hydrolase enzymes (Croux et al., 1993; Diaz et al., 1990; Diaz et al., 1991), reviewed by Lopez et al. (1997). These works illustrate the requirement of choline for binding by the choline binding domain, and how the binding domain can in turn control the lytic domain activity when present in a heterologous fusion. In contrast, no SH3b domains have been shown to be dependent on choline for binding. In fact, the exact binding site for the SH3b domain is still in question.

The most elegant SH3b cell wall binding studies have examined either lysostaphin or the nearly identical ALE-1 SH3b domain. Lu et al. have shown with site-directed mutagenesis that amino acid residues outside of the canonical 63 amino acid SH3b domain are important for optimal cell wall binding (Lu et al., 2006), while Grundling and Schneewind (2006) have studied the interaction between lysostaphin and staphylococcal peptidoglycan to identify the exact peptidoglycan binding site of lysostaphin.

The ability of the λSa2-E-LysK-SH3b or λSa2-E-Lyso-SH3b fusions to lyse both streptococci and staphylococci suggests that both LysK and lysostaphin SH3b cell wall binding domains harbor a generic or at least a dual-species peptidoglycan binding activity. These results indicate that a cell wall binding domain (e.g. SH3b domain or Cpl-7) from either streptococcal or staphylococcal origin could serve to increase both streptococcal and staphylococcal cell lytic activity when used in conjunction with this streptococcal endopeptidase domain that cleaves a peptidoglycan bond present in both species. An alternative explanation is that the λSa2-E lytic domain might be contributing sufficient substrate recognition in the fusion constructs to effectively recognize and digest the streptococcal peptidoglycan. Although the lytic domain might contribute to some cell wall binding, we consider this contribution minor and insufficient to fully explain these results due to the low level of lytic activity exhibited by the endopeptidase domain alone (λSa2-E construct). The enhanced streptolytic and staphylolytic activity of the staphylococcal fusion constructs (λSa2-E-Lyso-SH3b λSa2-E-LysK-SH3b) suggests that the staphylococcal SH3b domains both recognize and bind to both streptococcal and staphylococcal peptidoglycan. These results and the fact that streptococcal peptidoglycan lacks a pentaglycine bridge is in apparent conflict with the elegant cross-linking studies of Grundling and Schneewind (2006) that concluded "intact pentaglycine cross bridges are essential" for the binding of the lysostaphin cell wall targeting domain to peptidoglycan.

5. Conclusions

In our quest to identify the optimal staphylococcal SH3b_5 cell wall binding domain for chimeric antimicrobial proteins, we have categorized the candidate domains in the literature to eleven unique groups or proteins. Two subgroups were identified with overlapping but differentially conserved residues. The consensus residues conserved in previous multi-species alignments (Lu et al., 2006; Ponting et al., 1999) are not all present in the staphylococcal SH3b domain suggesting a potential genus-specific cell wall binding consensus sequence might exist and that warrants further analysis. We correctly predicted that a streptococcal lytic domain with weak staphylolytic activity could have its staphylolytic activity increased through fusion to a staphylococcal cell wall binding domain. Both the LysK and lysostaphin C-terminal sequences (including their respective SH3b domains) conferred increased staphylolytic activity to the LambdaSa2 endopeptidase domain. Interestingly, the heterologous fusions with staphylococcal SH3b domains did not eliminate streptolytic activity, suggesting some cross-species recognition of peptidoglycan by these staphylococcal SH3b domains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2009.04.023.

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